Low-dose low–molecular-weight heparin is anti-inflammatory during venous thrombosis

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Purpose: **Venous thrombosis results in a vein wall inflammatory response initiated by thrombus. Although anticoagulation with standard heparin (SH) and low–molecular-weight heparin (LMWH) is known to limit further thrombosis, their anti-inflammatory properties are poorly defined. The anti-inflammatory properties of these heparins were studied.**
Methods: **Sprague-Dawley rats were divided into groups and underwent inferior vena caval (IVC) ligation just below the renal level producing IVC thrombosis. One hour before ligation, animals received subcutaneous SH or LMWH at either high or low dose; normal saline (NS) was used as control. Six hours after ligation, animals were killed, and the IVCs were analyzed for clot presence, vein wall morphometrics, and vein wall permeability (VP) to define injury.**
Results: **Animals in both low-dose groups had no measurable anticoagulation, whereas those in both high-dose groups were adequately anticoagulated. There were statistically less IVC neutrophils for all groups compared with the control group, with low-dose LMWH showing the least cells (low-dose LMWH, 16 ± 3; high-dose LMWH, 37 ± 10; low-dose SH, 37 ± 6; high-dose SH, 32 ± 9; NS control, 63 ± 2). Similar results were noted for total inflammatory cells. The lowest VP was noted for low-dose LMWH.**
Conclusion: **Although both SH and LMWH inhibited vein wall neutrophils and total inflammatory cells, low-dose LMWH was most effective limiting neutrophil extravasation and was the only intervention to decrease VP below control levels. This occurred without preventing thrombus formation or causing a state of anticoagulation. Low-dose LMWH possesses anti-inflammatory properties distinct from its anticoagulant properties. (J Vasc Surg 1998;28:848-54.)**

Deep venous thrombosis and its subsequent sequelae of pulmonary embolism, chronic venous insufficiency, or both remain significant medical problems in our society. It has been estimated that 260,000 people each year are hospitalized in acute-care facilities for the treatment of venous thrombosis-related illness.[1] Previous work from our laboratory has demonstrated that venous thrombosis contributes to an active vein wall inflammatory response that is initiated and propagated by further thrombosis.[2] This response is characterized by early vein wall neutrophil infiltration followed by monocyte/macrophage infiltration. Although the exact mechanism that governs the thrombosis/vein wall inflammatory response has not been fully elucidated, there appears to be a complex regulatory mechanism, involving a balance between pro-inflammatory and anti-inflammatory vein wall cytokines, that regulates the intensity of the inflammatory response.[3] Concomitantly, platelet and endothelial cell derived adhesion molecules contribute to the initial reversible and subsequent irreversible influx and binding of these vein wall inflammatory cells to the endothelium, further propagating the inflammatory response.[4]

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Heparin is the anticoagulant traditionally used for the treatment of thromboembolic disease. It is known to possess multiple actions, including potent anticoagulant, antithrombotic, and antiplatelet activity.[5-7] Heparin also possesses anti-inflammatory activity that contributes to the relief of spasm and pain associated with thrombus propagation.[8,9] However, despite these findings, its vein wall anti-inflammatory activity has been poorly characterized. Characterized even less well is the anti-inflammatory activity for heparin fractions known as low–molecular-weight heparins. Therefore, in a well-described rodent model of stasis-induced venous thrombosis,[2] we set out to better define the anti-inflammatory effects of standard unfractionated heparin (SH) and low–molecular-weight heparin (LMWH) in the setting of thrombus-induced vein wall inflammation.

In this study, using both low and high doses of SH and LMWH, we found that both SH and LMWH significantly decreased the vein wall inflammatory response associated with stasis-induced venous thrombosis. Importantly, LMWH, at non-anticoagulant dosing, was the most effective, possessing anti-inflammatory properties that were separate from its anticoagulant activity.

MATERIALS AND METHODS

Animal model and tissue analysis. Sprague-Dawley rats (n = 96), weighing 250 to 300 grams, were randomly allocated to 5 separate groups and underwent inferior vena caval (IVC) ligation just below the level of the renal veins, as previously described.[2] In each group of animals, 6 underwent morphometric analysis, 6 underwent vein wall permeability studies, and 6 underwent thrombus weight analysis (except for the control group, in which an additional 6 underwent thrombus weight evaluation). Coagulation analysis was performed on those animals studied with morphometries and thrombus weight determinations in each group, including the additional 6 control rats noted above.

Animals were anesthetized by means of inhalation of isoflurane (1% to 2%) and oxygen (100%). Aseptic midline laparotomy was performed, and the thrombus was induced by ligating the IVC just below the level of the renal veins with concomitant ligation of IVC draining side branches. One hour before ligation, the animals within a group received a single subcutaneous injection into the abdominal wall of either high-dose SH (450 U/kg; Elk-Sinn, Cherry Hill, NJ), low-dose SH (300 U/kg), high-dose LMWH (450 anti-Xa U/kg; Fragmin, Pharmacia & Upjohn, Kalamazoo, Mich), or low-dose LMWH (100 anti-Xa U/kg). These doses of anticoagulants were chosen from previous studies in rats so the high dose would produce an anticoagulant effect, whereas the low dose would result in no measurable anticoagulant effect. A final group received normal saline (NS) subcutaneous injection at an equivalent volume 1 hour before ligation and served as a control group.

We began documenting the baseline level of rat serum anticoagulation by determining activated partial thromboplastin time (aPTT), thrombin clotting time (TCT), and anti-factor-Xa activity in NS-treated animals. Six hours after thrombus induction and while the rats were under general anesthesia, blood was drawn from the tail veins of the experimental animals, and the degree of serum anticoagulation was determined just before the rats were killed. Animals receiving SH underwent aPTT and TCT assays, whereas those undergoing LMWH treatment were monitored by means of aPTT, TCT, and anti-factor Xa assays. Control animals also underwent aPTT, TCT, and anti-factor Xa assays. All animals were subsequently killed under isoflurane anesthesia, and the infrarenal IVC below the ligature site was harvested, weighed, and grossly examined for the presence of thrombus. The harvesting of the IVC occurred simultaneously with or just before death. Vein wall specimens were used for either morphometric evaluation (n = 6) or vein wall permeability (n = 6) as a marker of vein wall injury.

Coagulation analysis. The aPTT assays were performed on 0.1 mL platelet-rich plasma with 0.1 mL rabbit cephaloplastin (Baxter, Miami, Fla) and 0.1 mL of 0.02 mol/L CaCl2 added as an activator on a fibrometer (Baxter). TCT samples were measured on a fibrometer with 0.02 mL platelet-rich plasma and 0.1 mL TCT reagent (American Dade, Miami, Fla). The aPTT and TCT measurements were performed in duplicate, and the results were averaged.

Anti-factor Xa measurements were performed after cold centrifugation (3000 rpm for 20 minutes at 4°C) of citrated blood stored on ice after collection. In the anti-factor Xa assay (Coatest Heparin, KB Virtum, Stockholm, Sweden), excess antithrombin, followed by factor Xa, was added to the plasma sample, which was then reacted with a chromogenic substrate S-2222.[10] Anti-factor Xa activity was inversely proportional to absorbency at 405 nm, which indicated the quantity of remaining uninhibited factor Xa, as a measure of residual LMWH activity. Concentrations were then determined from standard curves constructed from known amounts of LMWH and their corresponding absorbencies for the assay.

Histopathologic analysis and leukocyte morphometric analysis. For vein wall specimens undergoing vein wall morphometric analysis, the intact vein wall and clot was fixed in 10% formaldehyde for 24 hours and then placed into a 70% alcohol solution for subsequent staining with hematoxylin and eosin. Leukocyte vein wall trafficking and emigration analysis was performed by means of standard histologic analysis and morphometrics, as previously described.[2,3] After staining the paraffin-embedded slides with hematoxylin and eosin, morphometric analysis was performed under high power (oil emersion) light microscopy by counting each section of tissue for various inflammatory cell types. Each vein wall was assessed for the number and type of cells, including the thrombus vein wall interface and extending the width of 1 high power field (HPF, 1000×). Five HPF sections of each vein wall were analyzed. Cells were classified as neutrophils, monocytes or lymphocytes with standard morphologic criteria, including cell size, cytoplasm content, and nuclear size. Morphometric analysis has been previously correlated to cell type by means of immunohistochemical staining.[3]

Vein wall permeability. This technique was used to evaluate vein wall injury in response to stasis-induced venous thrombosis. Microvascular permeability was measured with a modification of a previously described technique.[11-14] Animals received 20 mg/kg of Evan's blue dye (Sigma Chemical, St. Louis, Mo) through tail vein injection 3 hours before being killed. When the rats were killed, the IVC length and weight was determined, then the thrombus was separated from the vein wall. Additionally, when the rats were killed, a heparinized blood sample was taken from the IVC, and blood cells were removed by means of centrifugation. After homogenizing the vein wall specimen for 10 seconds × 3 in phosphate buffered saline (PBS), the specimens were incubated at 60°C in formamide for 18 hours. Thereafter, the supernatant was separated by means of centrifugation at 5000 rpm for 30 minutes. Three IVC vein wall specimens were combined and pooled for analysis. Evan's blue in the supernatant was quantitated by means of dual wavelength spectrophotometric analysis at 620 nm and 740 nm. This method allows for correction of the absorbency of contaminating heme pigments with the following formula: corrected absorbency at 620 nm = actual absorbency at 620 nm – [1.426 (absorbency at 740 nm) + 0.03]. Vein wall permeability results were expressed as a ratio of absorbency of Evan's blue in the vein wall, normalized to length, compared with that in the blood.

Statistical evaluation and animal use. Mean ± SE and unpaired Student *t* test comparing experimental groups with control groups were used when appropriate. To determine if differences existed within treatment groups, 1-tailed analysis of variance (ANOVA) was performed. Individual *t* tests between groups were performed if ANOVA values suggested differences between the 5 groups. Significance was defined at a level of $P \leq .05$.

All animals used in this study were housed and cared for in the University of Michigan Unit for Laboratory Animal Medicine under the direction of a licensed veterinarian according to the "Principles of Laboratory Animal Care" (formulated by the National Society for Medical Research) and *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health [NIH] Publication No. 86-23, revised 1985). The protocol was approved by the University of Michigan Committee on the Use and Care of Animals.

RESULTS

Clot presence was lowest in the high-dose LMWH group. All animals underwent successful infrarenal IVC ligation. However, the high-dose LMWH group revealed a lower number of IVCs with clot (13 of 18 specimens positive for clot) as compared with the NS-treated animals (18 of 18 specimens positive for clot), low-dose LMWH group, high-dose SH group, and low-dose SH group (17 of 18 specimens positive for clot). Thrombi in both LMWH groups were grossly smaller than those in the SH or control groups.

Thrombus weight was lowest in the LMWH-treated animals. Thrombus weight in the NS controls averaged 53.9 ± 5.1 mg (n = 12), whereas in the high-dose SH and low-dose SH groups the thrombus weight averaged 60.6 ± 4.8 mg (n = 6) and 51.5 ± 3.1 mg (n = 6), respectively. In the high-dose LMWH and low-dose LMWH groups, these weights were 24.4 ± 5.6 mg (n = 6) and 39.8 ± 2.9 mg (n = 6), respectively. Significant differences between groups were found by ANOVA ($P < .01$), and high-dose LMWH weight was significantly different from all other groups ($P < .05$). Low-dose LMWH was also significantly different from both high-dose SH and low-dose SH ($P < .05$).

Adequate anticoagulation was achieved in animals treated with both high-dose SH and high-dose LMWH. Six hours after thrombus initiation, the rat serum anticoagulation profile (Table I) revealed little anticoagulation in the animals treated with low-dose SH, as compared with the NS-treated

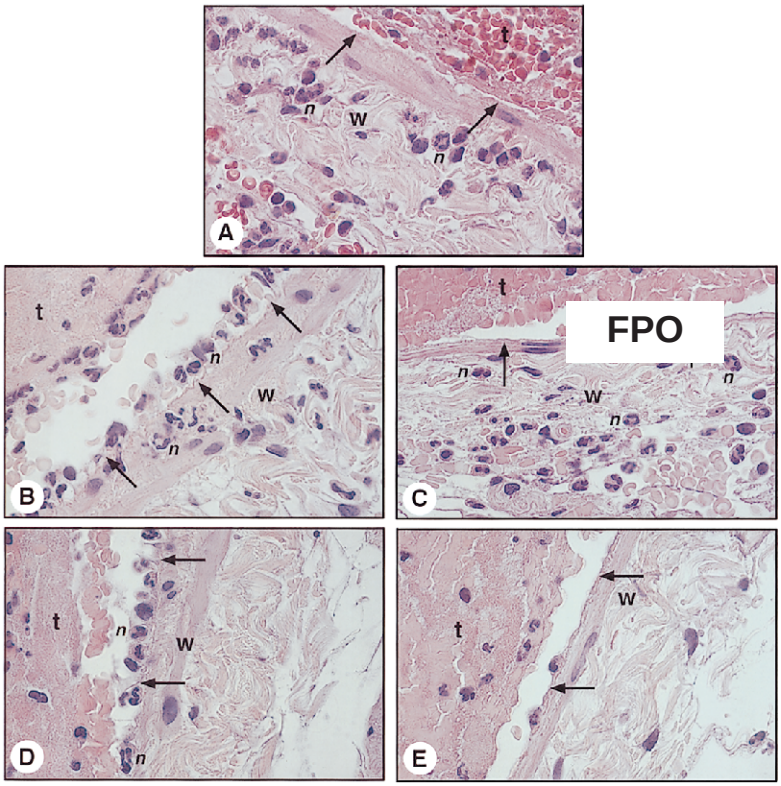

Fig 1. A, Inferior vena cava (IVC) of a control rat treated with normal saline; note presence of inflammatory cells (primarily neutrophils) in the IVC wall. **B,** IVC of a rat treated with high-dose heparin; note inflammatory cells (primarily neutrophils) at the luminal surface of the IVC and in the IVC wall. **C,** IVC of a rat treated with low-dose heparin; inflammatory cells (primarily neutrophils) again noted in IVC wall. **D,** IVC of a rat treated with high-dose low–molecular-weight heparin; inflammatory cells primarily at the luminal surface of the IVC. **E,** IVC of a rat treated with low-dose low–molecular-weight heparin; note lack of inflammatory cells at the luminal surface and in the IVC wall. *w,* wall of IVC; *n,* neutrophils; *t,* thrombus; *arrows* point to thrombus-vein wall interface. All 1000× (oil); Hematoxylin and Eosin.

group (low-dose SH aPTT 15.5 ± 0.6 seconds, TCT 22.3 ± 1.4 seconds; NS aPTT 12.3 ± 1.7 seconds, TCT 20.8 ± 1.0 seconds). However, at 6 hours, animals treated with high-dose SH were adequately anticoagulated (high-dose SH aPTT 51.8 ± 9.6 seconds, TCT 43.6 ± 8.4 seconds). Animals treated with low-dose LMWH revealed no anticoagulation via anti-factor-Xa activity, as compared with the NS-treated animals (NS = 0.32 IU/mL vs. Low-Dose LMWH = 0.30 IU/mL). However, animals treated with high-dose LMWH demonstrated adequate anticoagulation at 6 hours (high-dose LMWH = 1.09 IU/mL).

Vein wall inflammation, as assessed by morphometrics, was significantly less in anticoagulant-treated animals than NS controls (Fig 1). Differences by ANOVA were found between groups for neutrophils ($P < .01$) and total inflammatory cells ($P < .01$). The total leukocyte count of the infrarenal IVC wall in NS-treated animals revealed 82 ± 3 inflammatory cells per 5 HPF, with an absolute differential of 63 ± 2 neutrophils, 18 ± 1 monocytes, and 1 ± 1 lymphocytes (n = 6) (Table II). In the animals treated with high-dose SH, there was a 49% decrease in neutrophils ($P < .01$) and a 38% decrease in the total inflammatory cell count ($P < .05$). Animals treated with low-dose SH and high-dose LMWH also had a significant decrease in vein wall neutrophils ($P < .01$, $P < .05$) and total inflammatory cells (for low-dose SH, $P < .01$), as compared with the NS-treated animals. However, the greatest difference in vein wall inflammatory cell influx was seen in the animals treated with low-dose LMWH, with 34 ± 3 total inflammatory cells (59%

Table I. Anticoagulation profile

	aPTT (sec)	*TCT (sec)*	*anti-factor Xa activity (IU/mL)*
Normal saline	12.3 ± 1.7 (n = 8)	20.8 ± 1.0 (n = 11)	0.32 ± 0.38 (n = 18)
High-dose SH	51.8 ± 9.6 (n = 9)	43.6 ± 8.4 (n = 12)	
Low-dose SH	15.5 ± 0.6 (n = 10)	22.3 ± 1.4 (n = 12)	
High-dose LMWH	56.9 ± 13.1(n = 12)	75.8 ± 8.2 (n = 12)	1.09 ± 0.06 (n = 12)
Low-dose LMWH	16.0 ± 3.1 (n = 7)	21.2 ± 1.6 (n = 4)	0.30 ± 0.39 (n = 12)

aPTT, activated partial thromboplastin time; *TCT,* thrombin clotting time; *SH,* standard heparin; *LMWH,* low–molecular-weight heparin.

Table II. Vein wall morphometrics

	Neutrophils	*Monocytes*	*Lymphocytes*	*Total inflammatory cells*
Normal saline (n = 6)	63 ± 2	18 ± 1	1 ± 1	82 ± 3
High-dose SH (n = 6)	32 ± 9†	17 ± 3	2 ± 1	51 ± 12*
Low-dose SH (n = 6)	37 ± 6†	13 ± 1	2 ± 1	52 ± 6†
High-dose LMWH (n = 6)	37 ± 10*	18 ± 3	2 ± 1	57 ± 11
Low-dose LMWH (n = 6)	16 ± 3†‡	17 ± 1	1 ± 1	34 ± 3†‡

*P < .05 (compared with normal saline)
†P <.01 (compared with normal saline)
‡P < .05 (low-dose SH vs. low-dose LMWH)
SH, standard heparin; *LMWH,* low–molecular-weight heparin.

decrease; P < .01) and an absolute differential of 16 ± 3 neutrophils (75% decrease; P < .01), 17 ± 1 monocytes, and 1 ± 1 lymphocytes.

Vein wall permeability, as a marker of vein wall injury, was lowest in the animals treated with low-dose LMWH. Vein wall permeability was lowest in the low-dose LMWH group, although differences between the groups were not noted by ANOVA. The vein wall permeability (expressed in absorbency units [aU] and normalized to vein wall length) revealed 0.035 aU in NS-treated animals, as compared with 0.056 aU in animals treated with high-dose SH; 0.051 aU in animals treated with low-dose SH; 0.040 aU in animals treated with high-dose LMWH; and 0.025 aU in animals treated with low-dose LMWH.

DISCUSSION

Since its initial introduction, heparin has been found to be essential in the treatment of thromboembolic disease and has been suspected of possessing potent anti-inflammatory activity.[8,9] Unfractionated SH is derived from a complex linear polysaccharide of 60,000 to 100,000 d, which is degraded to a heterogeneous mixture of approximately 12,000 d.[15] LMWH is isolated from SH by means of gel filtration chromatography or differential precipitation with ethanol, and it possesses greater anti-factor Xa activity than anti-factor IIa activity.[15,16] Although the biologic effects of heparin are multiple, its anti-inflammatory properties have been poorly defined, and the anti-inflammatory effects of LMWH are even less well-defined.

The anti-inflammatory properties of both standard heparin and LMWH at both high- and low-dose subcutaneous administration were characterized in a rat model of stasis-induced venous thrombosis. The rat has been shown to serve as an appropriate model for investigating the effects of heparin, with results applicable to humans.[17] Current clinical practice targets an aPTT value of 1.5 to 3.0 times baseline values as the goal for adequate anticoagulation, whereas the ideal dosing for LMWH has been less clearly defined. In our current study, adequate anticoagulation was achieved in both high-dose SH and high-dose LMWH groups. This was demonstrated with a 6-hour aPTT 4.2 × baseline and TCT 2.1 × baseline for the high-dose SH group and an anti-factor Xa level of 1.09 IU/mL for the high-dose LMWH group. The coagulation profile of both low-dose SH and low-dose LMWH groups demonstrated no anticoagulation (Table I). The presence of clot was noted in all treatment groups. However, animals treated with high-dose LMWH demonstrated the least amount of clot presence (13 of 18 specimens), as compared with the remaining treatment groups (17 of 18) and the control animals (18 of 18). Furthermore, both

LMWH-treated groups had qualitatively and quantitatively less thrombus as measured by means of thrombus weight, as compared with the SH-treated groups. This suggests that LMWH has a greater antithrombotic effect than SH, even when it did not prolong any of the coagulation parameters measured, as seen in the low-dose LMWH group. Clinically, LMWH has previously been found to suppress prothrombin activation more effectively than unfractionated SH in patients treated for venous thrombosis.[18]

Previous work from our laboratory has characterized the vein wall inflammatory response in the rat during the presence of venous thrombosis.[2,3] This response occurs in a predictable leukocyte trafficking scheme, with early neutrophil influx (by 6 hours) and later monocyte/macrophage infiltration most prominent between 2 and 6 days. Our current findings demonstrate that SH and LMWH result in decreased vein wall inflammation, as assessed by means of morphologic criteria (Table II). The greatest decrease in vein wall neutrophils was seen with low-dose LMWH, despite no demonstratable anticoagulation, as noted earlier. Both neutrophil margination at the surface of the vein wall and emigration into the vein wall were inhibited. This suggests LMWH may possess anti-inflammatory activity that is separate from its anticoagulant activity. No differences in monocytes were noted between groups, despite heparin's known ability to bind to the surface of monocytes.[19-21] However, because of the early time-point of evaluation in this study (6 hours), effects on monocytes would not necessarily be expected.

In addition to its effects on vein wall leukocyte infiltration, vein wall permeability, as a marker of vein wall injury, was lowest in the animals treated with low-dose LMWH and highest in animals treated with low-dose SH and animals treated with high-dose SH. In fact, the only group to be below control value for vein wall permeability was the low-dose LMWH group. Unfractionated SH has been associated with increased capillary permeability, which may be associated with an increased tendency for microvascular bleeding.[22] However, LMWH has been shown to increase neither capillary permeability nor microvascular bleeding.[22] Our data supports these observations, especially at a low, nonanticoagulant dose for LMWH.

The events involved in stasis-induced venous thrombosis have been previously correlated with the expression of pro-inflammatory and anti-inflammatory cytokines.[3] However, in other studies from our laboratory using vein wall enzyme-linked immunosorbent assay protein extraction techniques, neither SH nor LMWH at high- or low-dose influenced the expression of vein wall cytokines (data not shown). This suggests that the anti-inflammatory effects of SH and LMWH may not be cytokine-mediated.

Finally, SH and LMWH administration are clinically associated with decreased symptoms of pain and spasm associated with thrombus formation. However, the specific mechanisms of SH and LMWH action on vein wall inflammatory inhibition are not completely understood. A number of heparin's anti-inflammatory mechanisms have been previously proposed, including its ability to inhibit neutrophil chemotaxis and phagocytosis in vitro, bind and inhibit various vasoactive products such as bradykinin and endotoxins that act as neutrophil chemoattractans,[23] inhibit neutrophil aggregation,[24] and modulate the activity of a number of inflammatory proteins including cell growth factors, angiogenic factors, and complement.[25,26] In addition, heparin oligosaccharides have demonstrated binding to leukocyte adhesion molecules (L- and P-selectins) and the competitive displacement of pro-adhesive cytokines from the endothelial cell surface contributing to acute inflammatory inhibition.[27,28] Our results confirm SH anti-inflammatory properties during stasis-induced venous thrombosis and demonstrate that LMWH possesses even more potent anti-inflammatory activity than SH. Such activity favors the use of LMWH for the treatment of venous thromboembolic disease.

CONCLUSIONS

Heparin and LMWH limit inflammatory cell extravasation into the vein wall in association with venous thrombosis. The greatest vein wall anti-inflammatory effect was seen with low-dose LMWH, in which its anti-inflammatory activity is separate from its measured anticoagulant activity. For this reason, LMWH may be more effective in the treatment of venous thrombosis.

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